

PARTIAL CHARACTERIZATION OF THE FLAVORS OF  
OXIDIZED BUTTEROIL<sup>1</sup>

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## SUMMARY

Butteroil was oxidized at 40° C. and distilled in a falling-film molecular still. The volatile flavor components were trapped in a U-tube cooled with liquid nitrogen, and the flavor was extracted from the trap contents with a few drops of petroleum ether. The flavor concentrate was then fractionated by gas phase chromatography, using butanediol succinate polymer as a stationary phase. Most of the compounds in the distillate were carbonyl compounds and were identified by their retention times in the gas chromatograph and by paper chromatography of their 2,4-dinitrophenylhydrazones. The fractions from the gas chromatograph were also incorporated into milk and butter and judged organoleptically. It was found that the carbonyl compounds produced in relatively large amounts made only a limited contribution to the oxidized flavor. The main contributor to the characteristic oxidized flavor seemed to be a minor component which was present in amounts too small to be detected by gas chromatography or paper chromatography. This component came out in the vicinity of octanal and  $\alpha$ -heptenal. This same flavor component can be found in the flavor concentrates from linseed, safflower, and herring oils. From the fatty acid composition of these oils, it appears that linoleic acid is the probable precursor of the component primarily responsible for the oxidized flavor. Reactions with various carbonyl reagents indicate that the compound responsible for the oxidized flavor is a carbonyl compound.

Reports in the literature on the flavors resulting from the autoxidation of milk and milk products agree that the main components of these flavors are carbonyl compounds (3-7, 13-17). However, the component or components directly responsible have never been definitely established. Many carbonyl compounds have been isolated and identified in the course of these investigations. They have been found to be saturated aldehydes, ketones,  $\alpha$ -unsaturated aldehydes, 2,4-dienals, and dicarbonyls. However, most investigators have not been able to reproduce the original flavors by addition of the compounds they identified. Forss, Pont, and Stark (5, 6) claimed that the cardboard flavor of skim-milk could be closely duplicated by  $\alpha$ -octenal,  $\alpha$ -nonenal, and 2,4-dienals. Recently, Day and Lillard (3) concluded from their investigation of the autoxidation of milk lipids that a complete spectrum of saturated and unsaturated carbonyl compounds was necessary to produce the flavors resulting from the oxidation. The different flavors were believed to arise from the various ratios and levels of these compounds in the fat.

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Unfortunately, most workers have been forced to make derivatives of the carbonyl compounds in order to separate and identify them. The 2,4-dinitrophenylhydrazine derivatives may be regenerated with levulinic acid (12), but this method is not entirely satisfactory. Thus, the only real test for the flavors, the organoleptic test, was largely lost at the beginning of the experiments. Recently, Forss *et al.* (7, 14) have investigated the fishy flavor sometimes found in dairy products. The flavors were concentrated by steam distillation and fractionated by gas phase chromatography, using silicone oil and Carbowax 400 as stationary phases. This permitted the flavor evaluation of the various flavor components. In this work, and further extension of it which Dr. Forss has kindly made available to us (8, 9), the flavors have been correlated with the carbonyl compounds present in the various fractions as follows: painty (pentanal and 2-pentenal), oily (hexanal, heptanal, 2-hexenal), mushroom (unknown), metallic (unknown), tallowy (octanal, nonanal, 2-octenal, 2,4-heptadienal), cucumber (2-nonenal). The fishy flavor arose from a combination of the metallic and tallowy components.

Although the experiments reported in the present paper were completed when the work of Forss *et al.* (7-9, 14) began to be reported, the methodology and results show many points of agreement. In the present experiments, the flavor was concentrated from the oxidized fat by molecular distillation, and the flavor concentrate was fractionated by gas phase chromatography. As a result of these experiments it appears that a few compounds of unknown composition with intense flavors are responsible for the major flavor characteristics of the oxidized milk fat.

#### MATERIALS AND METHODS

*Preparation and oxidation of the fats.* The butter was prepared in small stainless steel churns from sweet cream obtained from the University dairy. It was melted at 40-50° C. and centrifuged. The resulting butteroil was clear and dry and had a bland taste. The butteroil was oxidized in a large carboy at 40° C. open to the air and in diffuse daylight. The linseed, herring, and safflower oils were commercial drying oils. The crude herring oil was deodorized to remove volatile amines. The linseed, safflower, and deodorized herring oil were allowed to oxidize at 40° C. for 4-6 wk.

*Preparation of the oxidized flavor concentrate.* From one to four liters of butteroil were passed through a Rota-film molecular still at the rate of 500 ml/hr at a pressure of 1-10  $\mu$  Hg. The still was heated just enough to keep the butteroil from solidifying during its passage through the still (40-50° C.). The flavor distillate from the oxidized butteroil was caught in a U-trap immersed in liquid nitrogen. A second liquid nitrogen-cooled trap was placed between the first trap and the diffusion pump of the molecular still to protect the flavor concentrate from vapors from the diffusion pump. The distillate always contained some water, which was removed by extracting the distillate with a few drops of Skellysolve B. Organoleptic tests revealed that the components responsible for the flavors were found almost exclusively in the Skellysolve layer. Flavor concentrates of the other oxidized oils were prepared in a similar manner.

*Carbonyl reference compounds.* The saturated aldehydes, ketones, acrolein, and crotonaldehyde were obtained commercially.  $\alpha$ -Pentenal and  $\alpha$ -hexenal were prepared by the method of Harrison and Daubert (11).  $\alpha$ -Heptenal,  $\alpha$ -octenal, and  $\alpha$ -nonenal were prepared by Bedoukian's method (1). The 2,4-dinitrophenylhydrazones of these compounds were prepared by reaction with saturated 2,4-dinitrophenylhydrazine in 2 *N* acid. The hydrazones were purified by recrystallization, and their purity was established by their melting points and ultraviolet spectra.

*Gas chromatography.* The components of the flavor concentrates were resolved in a Podbielniak Chromacon apparatus. Most of the separations were carried out at 130-140° C. on a 6- to 10-ft. column packed with a butanediol succinate polymer on Chromosorb (2). This packing was selected because it did not contaminate the exit gas with strong flavors or carbonyl compounds. Helium at a flow rate of 37-43 ml/min was used as the carrier gas. Usually, 50  $\mu$ l. of the distillate in petroleum ether were injected into the apparatus with the aid of a micrometer syringe. The sample heater was usually set at 250-300° C. A tail pipe heater was installed to prevent the condensation of the components as they emerged from the apparatus.

The components emerging from the tail pipe of the gas chromatograph were treated by each of the following techniques:

(1) The fraction corresponding to each peak was received separately in 50 ml. of fresh pasteurized homogenized milk. These samples were examined organoleptically at appropriate dilutions by a tasting panel. (2) Each fraction was received separately into 4 ml. of fresh, pasteurized, homogenized milk, then two to three drops of the milk were mixed into 10 g. of fresh butter. The butter was examined by a tasting panel. (3) Each fraction was received into a few milliliters of 2,4-dinitrophenylhydrazine solution in 2 *N* HCl. The 2,4-dinitrophenylhydrazones were collected by filtration and extraction and analyzed by paper chromatography.

The carbonyl reference compounds whose flavors were examined were purified by gas chromatography and tasted in milk.

*Paper chromatography.* The techniques used were modifications of those of Gaddis and Ellis (10). The chromatograms were prepared from strips of Whatman No. 3 paper 9.5 in. long and tapered from 1 1/4 in. at the top to 3/4 in. at the bottom. The chromatograms were developed in test tubes. The following systems were used: (1). For the separation of the hydrazones into ketones, saturated aldehydes,  $\alpha$ -unsaturated aldehydes, 2,4-dienals, and dicarbonyls the paper was developed with *n*-pentane. (2). For  $C_1$  and  $C_5$  saturated aldehydes, the paper was impregnated with a kerosene fraction (b.p. 193-200° C.) and developed with methanol-ethanol-water 10:7.5:10. (3). For  $C_5$  to  $C_{10}$  saturated aldehydes,  $C_3$  and  $C_8$   $\alpha$ -unsaturated aldehydes, and ketones, the paper was impregnated with a 7% solution of vaseline in benzene and developed with methanol-ethanol-water 10:7.5:10. (4). For  $C_9$  to  $C_{12}$   $\alpha$ -unsaturated aldehydes and 2,4-dienals, the paper was impregnated with 7% solution of vaseline in petroleum ether (b.p. 91-95° C.) and developed with methanol-water 89:11.

*Flavor terminology.* In describing the flavors of single components and mixtures encountered in these experiments, the familiar terms which came closest to describing the flavors were used. Thus, the flavors associated with the early stages of oxidation in milk and butter (emery, cardboard, oxidized) were called oxidized. Flavors similar to the tallowy flavors which develop during the later stages of oxidation in milk and butter were called tallowy. Flavors which develop sometimes during the later stages of the oxidation of butter—painty, oily, and metallic—were used to describe the flavors of certain fractions. The painty flavor resembles the flavor of a drying oil such as linseed oil, though often the aroma is more characteristic of drying oils than the taste. The oily flavor resembles the plant-like, grassy flavor of an unrefined vegetable oil.

*Paint-like butteroil.* A sample butteroil which had been subjected to oxidation for 12-18 mo. was used in these experiments. The oil was completely bleached and had a paint-like, linseed oil aroma. A flavor concentrate of this butteroil was prepared and analyzed by gas phase chromatography, as previously described. The concentrate gave the characteristic pattern of peaks shown in Figure 1. The various components from the gas chromatography were examined organoleptically and by paper chromatography of their 2,4-dinitrophenylhydrazones. The results are recorded in Table 1.

The paint-like aroma characteristic of this sample was found in the second fraction, which gave a slightly oxidized flavor when tasted in milk and butter. The fraction was shown to contain pentanal and 2- or 3-pentanone, but the painty aroma and characteristic taste of this fraction could not be duplicated by mixtures of these carbonyl compounds. Thus, it was concluded that some other constituent of this fraction must be responsible for the painty aroma.

Fraction 4A gave a flavor in milk similar to that associated with the early stages of oxidation, and also gave a rather typical oxidized flavor in butter. This fraction contained  $\alpha$ -pentenal; however, it is doubtful if this component was responsible for the flavor, since some samples which contained  $\alpha$ -pentenal did not yield an oxidized flavor.

Fractions 5, 6A, and 9B were at times judged to have flavors reminiscent of various types of oxidation in milk fat.

Fraction 10B and subsequent fractions had a strong, oily flavor. Probably this high-boiling compound condensed partially in the tail pipe and contaminated subsequent fractions.

Fraction 6B gave a typical oxidized flavor to both milk and butter. When diluted, it gave a flavor which was quite similar to that of the early stages of oxidation in both milk and butter. In heavier concentrations, it resembled a tallowy flavor. This interesting flavor component appeared in the region between octanal and  $\alpha$ -heptenal, but synthetic  $\alpha$ -heptenal and octanal, and mixtures of these compounds, did not resemble the oxidized flavor. Indeed, it was found that oxidized flavor masked the flavor of additional octanal and  $\alpha$ -heptenal added to the flavor distillate. Thus, the flavor seems to be caused by some minor component with an extremely intense flavor. No observable peak for this component has ever been found with the gas chromatograph.

# FLAVORS OF OXIDIZED BUTTEROIL

After 18 mo. of storage, the flavor and aroma of this painty butteroil began to change in character, although the pattern of peaks obtained on the gas chromatograph of the flavor distillate showed little change. Eventually, this lot of butteroil no longer yielded the characteristic painty aroma found in Fraction 2. Also, Fraction 4A no longer yielded an oxidized flavor. The typical oxidized flavor in Fraction 6B decreased in intensity, but the oily flavor characteristic of Fraction 10B remained quite pronounced.

*Tallowy butteroil.* Butteroil which had been oxidized for a few months

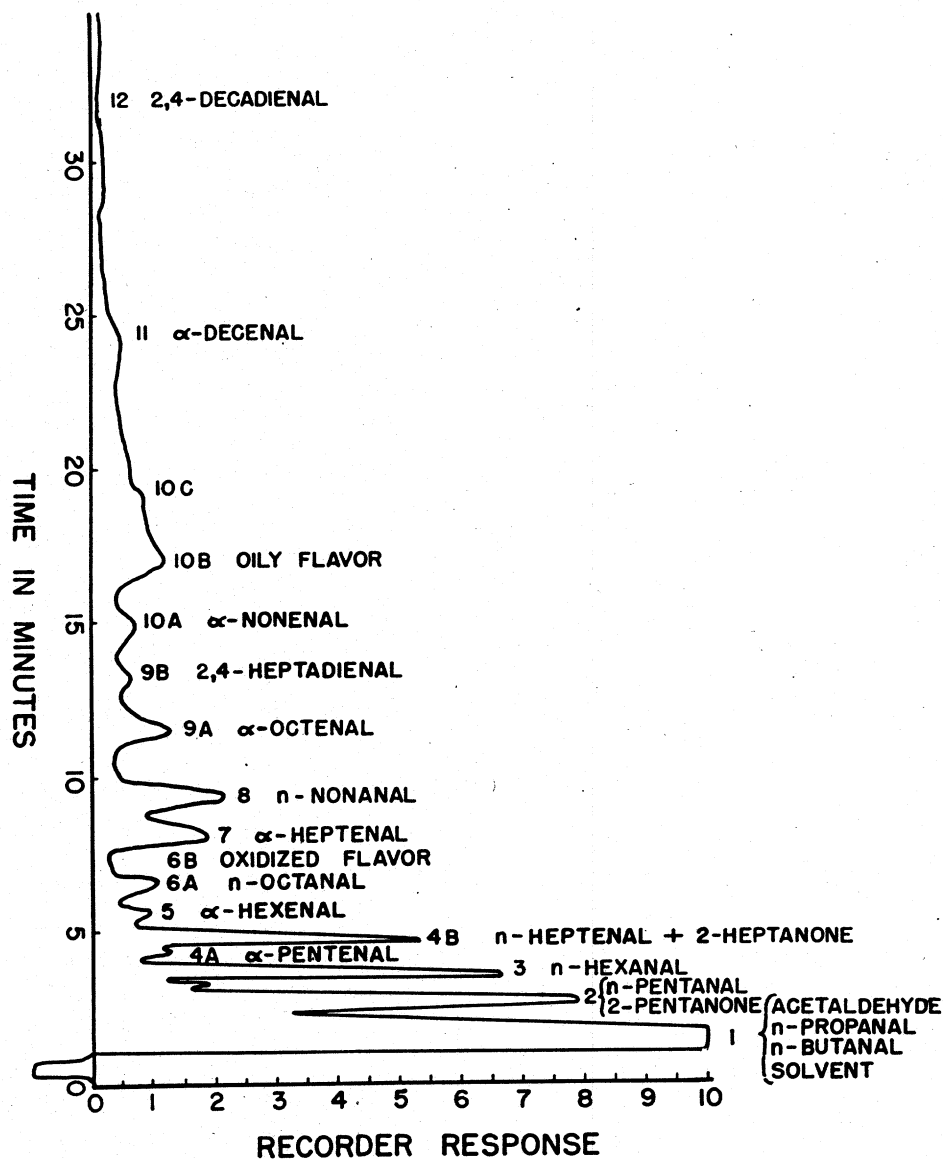


FIG. 1. Gas chromatography chart of the flavor concentrate from painty butteroil.

yielded a flavor concentrate which gave a typical tallowy flavor to milk. When analyzed on the gas chromatograph, it gave much less total carbonyl compounds than the previous sample. There was no painty aroma in Fraction 2 or oxidized flavor in 4A. However, Fraction 6B still had a pronounced oxidized flavor and a fraction in the vicinity of 10B gave a strong, oily flavor. When all the fractions received from the tail pipe of the gas chromatograph were placed in a single sample of fresh milk, the milk acquired a tallowy flavor, characteristic of the whole distillate. The fractions were divided into three groups: A containing all fractions up to 6B; B containing 6B; and C containing fractions after 6B. In milk, Fraction A by itself tasted like hexanal, its major constituent. Fraction B gave a flavor typical of early oxidation; the strong, oily flavor was predominant in C. Mixtures of A and C gave a flavor typical of an old, oxidized fat no longer edible. The stage of oxidation seemed to be a function of the ratio of B to A + C. High ratios of B to A + C gave a flavor resembling that of the early stages of oxidation in milk. Low ratios resembled a more advanced (tallowy) stage of oxidation.

*Tallowy butter.* A sample of tallowy butter which had been oxidized in cold storage for about 1 yr. was converted to butteroil and the flavor was concentrated. The flavor concentrate had an odor resembling Blue Mold Cheese. Gas chromatographic analysis also revealed that the concentrate had a higher concentration of ketones than did concentrates previously obtained from butteroils oxidized at higher temperatures. The oxidized flavor of Fraction 6B was again encountered, as was the oily flavor of 10B.

A batch of fresh butter was distilled as a control and was subsequently investigated by gas chromatography. The same pattern of peaks was obtained as in the painty butter, except that the peaks were relatively much smaller. A slight oxidized flavor was detected in a fraction corresponding to 6B.

*Resolution of the flavors on silicone columns.* Since the butanediol succinate polymer is a relatively polar stationary phase, it seemed wise to try gas chromatography on a nonpolar phase like silicone oil. These investigations were carried out on 6- to 10-ft. columns at 130° C. On this column, the relative positions of  $\alpha$ -heptenal and octanal are reversed from their order on butanediol succinate; however, the oxidized flavor which had been found in Fraction 6B, still came out between these two components. The fractions resulting from the silicone oil packing were contaminated with some foreign flavor components which appeared to be generated by this type of packing.

*Isolation of the oxidized and oily flavors from other fats.* To find the precursor of the oxidized flavor and a clue to its structure, flavor concentrates were prepared from oxidized linseed, safflower, and herring oils. A component similar to Fraction 6B from oxidized butteroil was found in the flavor concentrates from all of these fats. This component had a retention time between that of octanal and  $\alpha$ -heptenal and gave a typical oxidized flavor to milk. All these oils yielded the oily flavor encountered in oxidized butteroil. The component had a retention time close to that of undecanal. Several fractions from fish oil gave fishy flavors to the milk. The oils all gave hexanal, but there was little

TABLE 1  
Characterization of carbonyl compounds from the distillate of butteroil with a paint-like aroma

Fraction No.	Retention time in cm. (25.4 mm/2 min chart speed)* Flow rate = 40 ml/min		R <sub>f</sub> values on paper chromatograms		Revealed identity	Flavor in milk	Flavor in butter
	Unknown	Known	Unknown	Known			
1 <sup>b</sup>	1a	.....					
		2.3 and shoulder at 3.00					
	1b		0.63	0.65	Acetaldehyde		
	1c		0.52	0.50	n-Propanal		
2 <sup>c</sup>	2a	2.40	0.36	0.37	n-Butanal		
	2b	2.85	0.50	0.50	n-Pentanal	Painty aroma	Slight oxid.
		3.40	0.33	0.30	2-Pentanone		
3		3.75	0.36	0.38	n-Hexanal		
4A		4.4	0.53	0.52	α-Pentenal	Oxid.	Oxid.
4B <sup>e</sup>	4Ba	5.40	0.25	0.24	α-Heptenal		
	4Ba	5.90	0.21	0.26	2-Heptanone		
5		7.15	0.38	0.40	α-Hexenal		
6A		8.20	0.21	0.22	n-Octanal		Cod liver oil
6B		.....			Trace carbonyl		Metallic
7 <sup>d</sup>		10.10	0.30	0.30	α-Heptenal	Typical oxid.	Typical oxid.
8		11.40	0.14	0.13	n-Nonanal		
9A		14.40	0.22	0.23	α-Octenal		
9B		16.60	0.68	0.70 <sup>e</sup>	2,4-Heptadienal		Oxid.
10A		18.60	0.63	0.62	α-Nonenal		
10B		20.70			Dicarbonyl	Oily	Oily
10C		21.70	0.60	.....	α-Unsaturated		
11		31.20	0.53	0.55 <sup>e</sup>	α-Decenal		
12		50.90	0.62	0.60 <sup>e</sup>	2,4-Decadienal		

\* The large amount of solvent causes the peaks in the unknown to move faster than in the standard mixture of carbonyl compounds.

<sup>b</sup> Obscured by peaks from Skellysolve B.

<sup>c</sup> Other investigators found the 2-ketones in milk, and we have assumed on this basis that the ketones here are the 2-isomers (3).

<sup>d</sup> α-Heptenal was also identified by its u.v. and i.r. spectra, m.p., and mixed m.p. with synthetic α-heptenal.

<sup>e</sup> R<sub>f</sub> values are from Gaddis and Ellis (10). The system used reproduced their R<sub>f</sub>'s exactly on known compounds.

$\alpha$ -heptenal or octanal. None of the oils gave the painty aroma found in painty butteroil.

*The carbonyl nature of the oxidized flavor.* The carbonyl nature of the oxidized flavor compound was established as follows:

1. Upon reacting the flavor distillate from oxidized butteroil with several carbonyl reagents such as hydroxylamine, semicarbazide, 2,4-dinitrophenylhydrazine, and Girard's reagent T, the odor of the distillate was either altered substantially or removed entirely.

2. Some of the flavor concentrate from oxidized butteroil in Skellysolve B was shaken overnight with an aqueous solution of semicarbazide in acetate buffer at pH 4. The Skellysolve layer was recovered and examined by gas chromatography. It was found that the level of oxidized flavor in Fraction 6B was reduced to  $\frac{1}{5}$  to  $\frac{1}{10}$  of that of a control shaken with acetate buffer. The peaks representing octanal and  $\alpha$ -heptenal were obliterated. In a similar experiment, using Girard's reagent T in methanol, the oxidized flavor in 6B was almost completely removed, whereas a control left in methanol still possessed a strong flavor.

3. The flavor concentrate from oxidized butter was reacted with a few drops of mercaptoacetic acid for 15 to 20 min. and the reaction mixture was diluted with water and extracted with Skellysolve B. The oxidized flavor of Fraction 6B was completely absent in the Skellysolve extract.

Although these experiments indicate that the oxidized flavor is a carbonyl compound, it was not possible to find any indication of the oxidized flavor component of Fraction 6B among the 2,4-dinitrophenylhydrazones; only octanal and  $\alpha$ -heptenal could be detected by paper chromatography. There was some indication that a dicarbonyl might be present. The fraction from the gas chromatography which contained the oxidized flavor was collected in carbon tetrachloride. An infrared spectrum of this solution showed mainly  $\alpha$ -heptenal. The 2,4-dinitrophenylhydrazones prepared from the gas chromatograph fraction containing the oxidized flavor did not yield any oxidized flavor when they were regenerated (13). A typical  $\alpha$ -heptenal odor was produced.

#### DISCUSSION

Table 1 and Figure 1 show the main components identified to be saturated aldehydes, saturated ketones,  $\alpha$ -unsaturated aldehydes, 2,4-dienals, and dicarbonyls. These types of compounds have previously been identified in oxidized fats (3, 5, 6). They are essentially those found by Forss *et al.* (7) in fishy milk fat. Probably some of the short-chain carbonyl compounds were lost in removal of the water from the butter or flavor distillate. However, the components which were readily identified seem to make a limited contribution to the flavor. Fraction 6B by itself gives a flavor which is quite typical of the first detectable stages of oxidation in milk and butter. Apparently, as the oxidation progresses, other flavor components blend with 6B to give the sensation of more advanced stages of oxidation. Among the most important flavors in this blending process, the oily flavors in 10B and hexanal seem to predominate.



Hexanal was always found in higher concentration than the other carbonyl compounds. It is probably an important contributor to the flavor.

The painty flavor and aroma arising in butteroil in a certain stage of the oxidation process seem also to be due largely to a single component. It seems to be similar to the painty compound reported by Forss *et al.* (8, 9); however, it is evidently not due to pentanal and  $\alpha$ -pentenal, as they believed. Like 6B, it seems to be a minor component with intense flavor.

The metallic flavor component reported by Forss *et al.* (7, 8) resembled the flavor in 6B which we have designated oxidized, in that they have similar retention times on silicone columns and both are minor components with very intense flavors. However, a sample generously supplied by Forss had a metallic flavor completely different from any encountered in the present experiments. Probably the presence of copper is necessary for the production of metallic flavors, and copper was excluded from samples in these experiments. The flavor in 6B seems to correspond more closely to the tallowy components found by Forss *et al.* (7, 9). Their tallowy fraction contained octanal and should contain most of the fraction corresponding to 6B.

However, there are several differences in the present work and that reported by Forss *et al.* which are difficult to reconcile. These investigators report that cardboard flavors similar to those associated with the early stages of oxidation can be simulated with  $\alpha$ -octenal and  $\alpha$ -nonenal (5, 6), and that synthetic octanal and nonanal impart a tallowy flavor to butter (7). From the present experiments with the flavor concentrates and synthetic carbonyl compounds, it appears that Fraction 6B is a necessary ingredient in both the early (cardboard) and late (tallowy) stages of the flavor of oxidized milk lipid. The present experiments suggest no relationship between the saturated and  $\alpha$ -unsaturated  $C_8$  and  $C_9$  aldehydes and cardboard or tallowy flavors. Forss *et al.* (7) described the flavor of hexanal and heptanal as oily. These or other compounds issuing from the gas chromatograph in the vicinity of hexanal and heptanal were not found to give oily flavors in our experiments. Moreover, a flavor issuing from the gas chromatograph in the vicinity of undecanal was invariably described as oily or grassy. Probably the term oily is used to describe two different flavors in these instances.

The oxidized flavor in 6B could also be isolated from linseed, safflower, and herring oils. The only unsaturated fatty acids known to be common to all these oils are linoleic and oleic acids. The former is probably the precursor of the oxidized flavor.

The reactions between the oxidized flavor component and the various carbonyl reagents indicate that the oxidized flavor in 6B is a carbonyl compound. Presumably, it contains only oxygen, hydrogen, and carbon. A number of different carbonyl compounds have been investigated on silicone and butanediol succinate columns. These experiments showed that monocarbonyl compounds with additional oxygen functions (such as epoxide, alcohol, or dicarbonyls) show a much longer retention time on butanediol succinate than on silicone. In view of this finding, it seems unlikely that the flavor in 6B contains any oxygen-containing

functional group besides carbonyl. The flavor compound must be closely related to octanal or  $\alpha$ -heptenal. Attempts to synthesize  $\beta$ -unsaturated aldehydes were not successful. Possibly, the flavor compound contains a branched chain or an isolated double bond. The synthetic  $\alpha$ -heptenal is presumably the *trans* isomer. The *cis* form might be responsible for the oxidized flavor. It is tempting to speculate that the painty flavor in Fraction 2, the oxidized flavor sometimes found in 4A, and the oxidized flavor in 6B and perhaps 9B form an homologous series.

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